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(71) Applicant: STERLING WINTHROP INC. [US/US]; 90 Park Avenue, New York, NY 10016 (US).

(72) Inventors: COOK, Phillip, D.; 7340 Bolero, Carlsbad, CA 92009 (US). DELECKI, Daniel, J.; 141 Upper Gulph Road, Radnor, PA 19087 (US).

(74) Agent: HAKE, Richard, A.; 343 State Street, Rochester, NY 14650-2201 (US).

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(54) Title: 7-DEAZAPURINE MODIFIED OLIGONUCLEOTIDES

(57) Abstract

Oligonucleotides, which incorporate 7-deazanucleosides, are useful as antisense sequences to inhibit the function of RNA and DNA.

7-DEAZAPURINE MODIFIED OLIGONUCLEOTIDES

Field of the Invention

This invention relates to modified oligonucleotide sequences containing 7-deazapurine nucleosides, to a method of inhibiting nuclease degradation of oligonucleotides incorporating the same, to a method of inhibiting gene expression in a cellular system and to compositions useful for inhibiting gene expression containing the modified oligonucleotides.

Information Disclosure Statement

Seela and Kehne, Biochem., 26, 2232-2238 (1987) disclose 7-deazadeoxyadenosine (9-β-2'-deoxyribofuranosyl-7-deazaadenine) and the incorporation of from one to two such nucleosides into octa and dodecanucleotides having the palindromic EcoRI endonuclease DNA recognition sequence d(GAATTC). The oligonucleotides were prepared for study of their stability to cleavage by EcoRI.

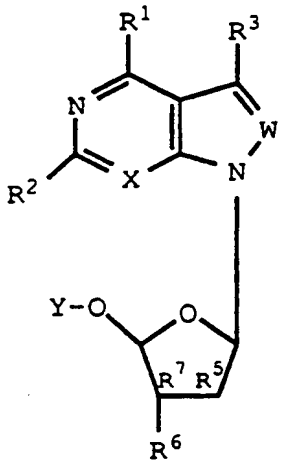
Seela and Driller, Nucl. Acid. Res., 17(3), 901-910 (1989) describe the preparation of hexanucleotide sequences containing $d(GC)_3$ and $d(CG)_3$ nucleotide units and such hexamers containing 7-deazaguanosine (c^7G_d) and 7-deaza-8-azaguanosine ($c^7z^8G_d$) nucleoside units. The self-complementary hexamers so-prepared form duplexes which were prepared for the purpose of studying the stability of the duplexes and the thermodynamic parameters of helix-coil transition for each of the G-C/C-G base pairs.

Tran-Thi et al., Angew. Chem. Int. Ed. Engl., 21(5), 367-368, (1982) disclose the preparation of 7-deaza-guanosine and the preparation therefrom of cyclic guanosine monophosphate.

Seela and Kehne, Biochem., 24(26), 7556-7561 (1985) describe the synthesis of self-complementary hexamers and dodecamers employing solid phase techniques

Seela, Tran-Thi and Franzen, Biochem., 21, 4338-4343 (1982) disclose the preparation of polymers of 7-deazaguanosine for study of their hypochromicity, melting profiles and circular dichroism spectra.

EPO Application 286,028, published October 12, 1988, discloses 7-deazapurine nucleosides of the formula:



where:

X is N or a =CH group;

W is N or a $=CR^4$ group;

R^1, R^2, R^3 and R^4 are the same or different hydrogen, halogen, lower-alkyl, hydroxy, mercapto, lower-alkylthio, lower-alkoxy, arylalkyl, arylalkoxy, aryloxy or a mono or di-substituted amino group;

R^5 is hydrogen or hydroxy;

R^6 and R^7 are hydrogen or one or both can be halogen, cyano, azido or a mono or di-substituted amino group, and wherein one of R^6 and R^7 can be hydroxy when X is a =CH group and furthermore R^5 and R^7 together can be a second bond between the C_2' and C_3' positions and Y is hydrogen or a mono, di or triphosphate.

The compounds are stated to be useful in nucleic acid sequencing, and as antiviral agents

necessitating the use of large, frequently administered, dosages.

Another problem is the extremely high cost of producing antisense DNA or RNA using available semiautomatic DNA synthesizers.

A further problem relates to the delivery of antisense agents to desired targets within the body and cell. Antisense agents targeted to genomic DNA must gain access to the nucleus (i.e. the agents must permeate the plasma and the nuclear membrane). The need for increased membrane permeability (increased hydrophobicity) must be balanced, however, against the need for aqueous solubility (increased hydrophilicity) in body fluid compartments such as the plasma and cell cytosol.

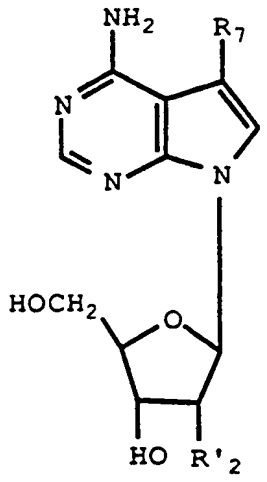
A still further problem relates to the stability of antisense agents whether free within the body or hybridized to target nucleic acids. Oligonucleotide sequences such as antisense DNA are susceptible to steric reconfiguration around chiral phosphorus centers.

Gene targeting via antisense agents is the predicted next step in human therapeutics [Armstrong, Business Week March 5, 1990, page 88]. The successful application of antisense technology to the treatment of disease, however, requires finding solutions to the problems set forth above.

One approach to preparing antisense compounds that are stable, nuclease resistant, inexpensive to produce and which can be delivered to and hybridize with nucleic acid targets throughout the body is to synthesize oligonucleotide sequences having incorporated therein modified adenine or guanine purine bases which are capable of hybridizing with their complementary respective thymine or cytosine bases but which are less susceptible to attack by exo- or endonucleases and which thus stabilize the oligonucleotide sequences to enzymatic degradation This invention is directed to such an approach.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

More specifically this invention relates to oligonucleotides incorporating a sequence of nucleotides of the normal DNA bases, i.e. adenine, thymine, guanine and cytosine, in the required sequence for hybridization with a given DNA or RNA base sequence and in which one or more of the normal bases are replaced by a 7-deazaadenine-β-D-ribofuranosyl or β-D-2′-deoxyribofuranosyl nucleoside of the formula:



Ia

or a 7-deazaguanine-β-D-ribofuranosyl- or β-D-2′-deoxyribofuranosylnucleoside of the formula:

Particularly preferred oligomers are those which incorporate nucleotides derived from the nucleosides of formula Ia wherein:

R'_2 and R_7 are hydrogen, i.e. 7-deaza-2'-deoxyadenosine (9-β-D-2'-deoxyribofuranosyl-7-deazaadenine), hereinafter identified as nucleotide W;

R'_2 is hydroxy and R_7 is hydrogen, i.e. 7-deazaadenosine (9-β-D-ribofuranosyl-7-deazaadenine), hereinafter identified as nucleotide W';

R'_2 is hydrogen and R_7 is lower-alkyl, i.e. 7-deaza-2'-deoxy-7-methyladenosine (9-β-D-2'-deoxyribofuranosyl-7-deaza-7-methyladenine), hereinafter identified as nucleotide X; and

R'_2 is hydroxy and R_7 is lower-alkyl, i.e. 7-deaza-7-methyladenosine (9-β-D-ribofuranosyl-7-deaza-7-methyladenine), hereinafter identified as nucleotide X';

and nucleosides of formula Ib wherein:

R'_2 and R_7 are hydrogen, i.e. 7-deaza-2'-deoxyguanosine (9-β-D-2'-deoxyribofuranosyl-7-deazaguanine), hereinafter identified as nucleotide Y;

R'_2 is hydroxy and R_7 is hydrogen, i.e. 7-deazaguanosine (9-β-D-ribofuranosyl-7-deazaguanine), hereinafter identified as nucleotide Y';

R'_2 is hydrogen and R_7 is lower-alkyl, i.e. 7-deaza-2'deoxy-7-methylguanosine (9-β-D-2'-deoxyribofuranosyl-7-deaza-7-methylguanine), hereinafter identified as nucleotide Z; and

R_2 is hydroxy and R_7 is lower-alkyl, i.e. 7-deaza-7-methylguanosine (9-β-D-ribofuranosyl-7-deaza-7-methylguanine), hereinafter identified as nucleotide Z'.

As used herein the term lower-alkyl means a saturated, aliphatic, straight or branched chain hydrocarbon radical containing from one to four carbon atoms and thus includes methyl, ethyl, propyl, isopropyl and butyl.

103, 3185-3191 (1981) and Gait, Oligonucleotide Synthesis:A Practical Approach , Ed. by M.J. Gait, 35-81, IRL Press, Washington, D.C. 1984.

The initial step in solid phase synthesis is attachment of a nucleoside to a solid support, preferably a controlled pore glass (CPG) support. The nucleoside is preferably attached to the CPG via a succinate linkage at the 3'-hydroxy position of the nucleoside. Other means of attaching nucleosides to solid supports are known and readily apparent to those skilled in the oligonucleotide synthesis art.

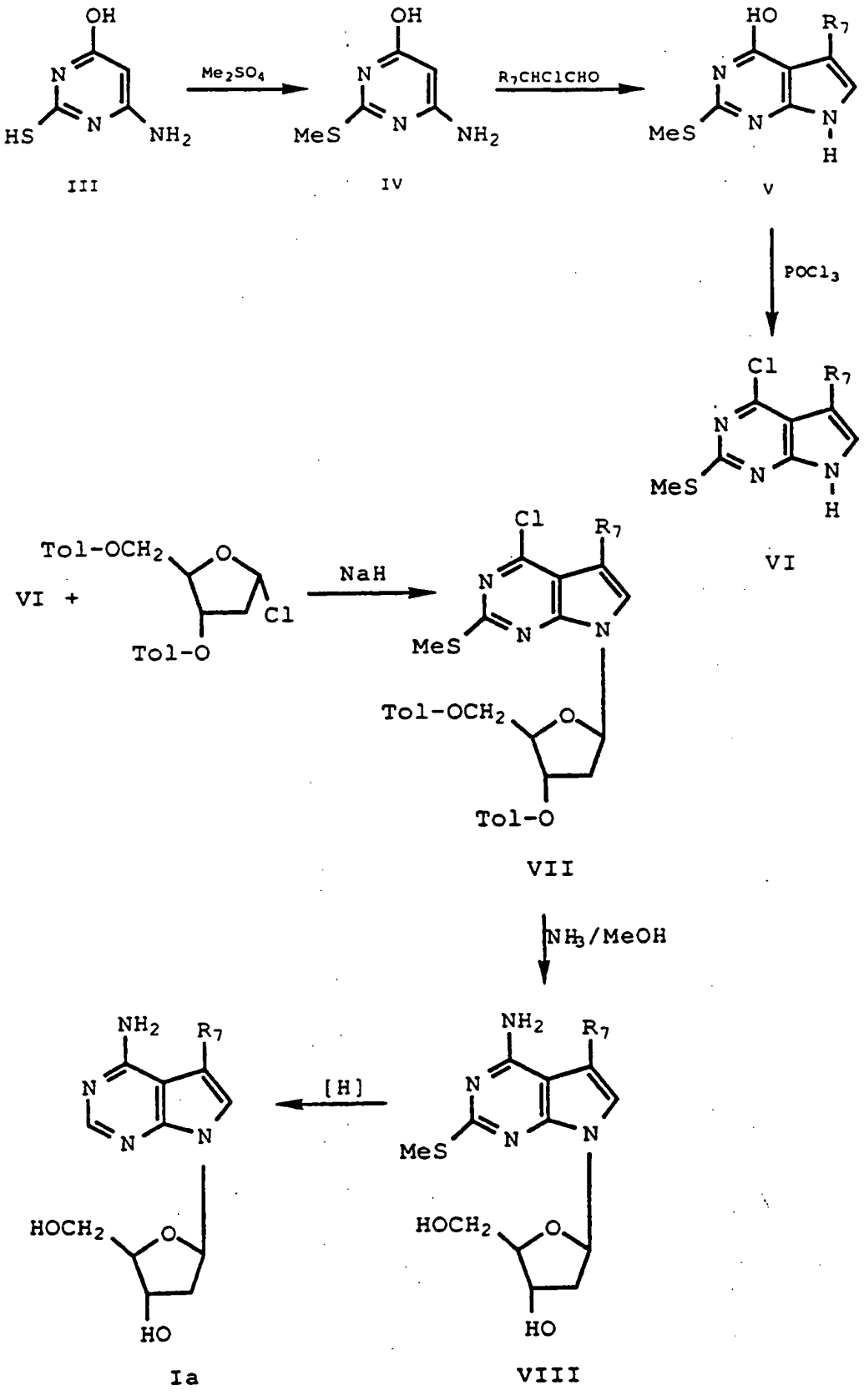
Following attachment of the first nucleoside to the solid support, chain elongation occurs via the sequential steps of removing the 5'-hydroxy protecting group, activating the 5'-hydroxy group in the presence of a phosphoramidite reagent, adding the desired nucleoside, capping the unreacted nucleoside and oxidizing the phosphorus linkage. The protecting group, preferably DMT, at the 5'-hydroxy position of the attached nucleoside is removed with acid, preferably trichloroacetic acid.

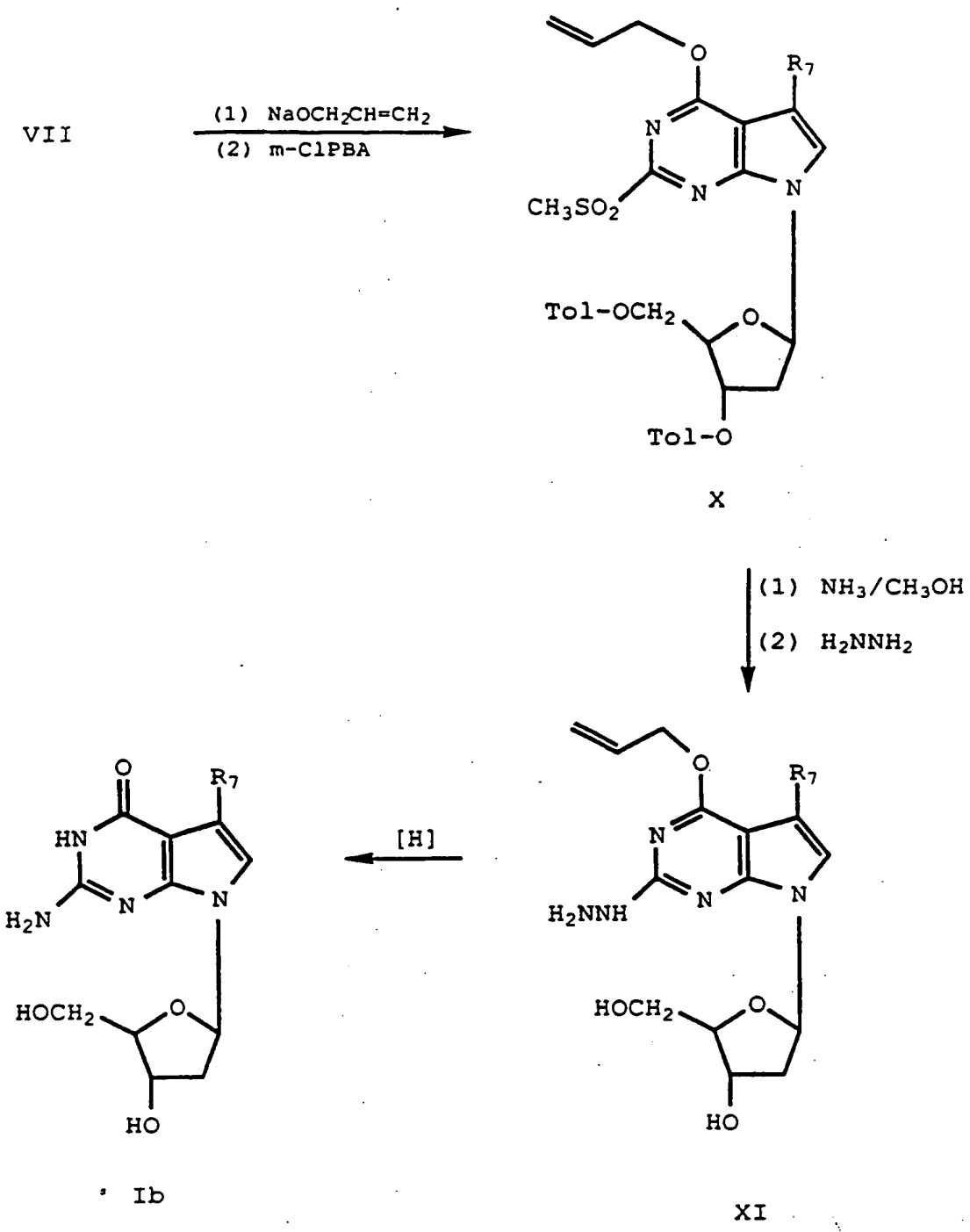
Activating reagents that can be used in accordance with this method are well known to those skilled in the art. Preferred activating reagents are tetrazole and activator gold (Beckman Instr. Inc., Palo Alto, CA).

The activation step occurs in the presence of the added nucleoside and a trityldiolcyanophosphine compound, which compound replaces the nucleoside phosphoramidite of conventional synthetic methods. Unreacted chains are terminated or capped with capping reagents such as acetic anhydride and N-methylimidazole.

The labile trivalent phosphorus linkage is oxidized, preferably with iodine, to the stable, pentavalent phosphodiester linkage of the oligonucleotide.

After the desired oligonucleotide chain assembly is complete, the phosphate protecting groups are removed, the chains are separated from the solid support and the base protecting groups are removed by conventional methods. (Gaits, supra at 67-70.)


OH
N
HS
N
NH2
Me2SO4
MeS
R7CHClCHO
HO
R7
H
III
IV
V
POCl3
Cl
VI
Tol-OCH2
VI +
Tol-O
NaH
VII
NH3/MeOH
[H]
HOCH2
HO
Ia
VIII



These can then be converted to the 9-β-D-2′-deoxyribofuranosyl-7-deaza-7-lower-alkylguanines of formula II, as described before.

The pharmaceutical compositions of the present invention include one or more of the compounds of this

targeted delivery systems such as polymer matrices, liposomes, and microspheres. They may be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders, as for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example, paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

The molecular structures of the compounds were established on the basis of a study of the nmr, infrared and mass spectra, and their purities were established by HPLC and chemical analysis for their elements.

Nuclease Stability

Antisense oligonucleotides modified in accordance with the present invention were evaluated (and compared against unmodified oligonucleotides) for their stability in the presence of 10% (v/v) fetal bovine serum

polyacrylamide gel electrophoresis. The 15 cm gels were prepared using electrophoresis buffer (0.1 M sodium phosphate, pH 7.2 containing 1.0 g of SDS/L) and contained 12.5% acrylamide and 0.6% bisacrylamide. Aliquots (1 uL) of translation reactions were diluted with 11 uL of loading buffer consisting of electrophoresis buffer, 1.1% 2-mercaptoethanol, 2.5% glycerol and bromphenol blue. Samples were denatured by heating to 100°C for 3 min. before loading onto gels. The gels were run for 18 hours at 30 mAMP. After electrophoresis, gels were stained with coomassie blue, dried and autoradiographed at -70°C for 16 hours.

Quantitation of the effects of alpha globin directed antisense oligonucleotides on the synthesis of alpha globin was done by scanning the autoradiographs using an Ultrascan XL laser densitometer (LKB/Bromma) linked to an AT&T PC6300 computer. Data were collected, displayed and integrated with the Gelscan XL data analysis software package (LKB/Bromma). Effects of oligomers on protein synthesis were expressed as a percent of control alpha globin synthesis.

The following examples will further illustrate the invention without limiting it thereto. It will be apparent to those skilled in the art that the embodiments disclosed may be readily modified by standard procedures to produce oligonucleotides of other lengths and with other sequences. Targets for synthesis will usually be chosen by substituting a 7-deazaadenine or 7-deazaguanine nucleoside of formulas Ia or Ib in the sequence which is to be protected from nuclease degradation or which is complementary to a sequence which is to be blocked.

a few minutes. The mixture was treated with about 0.5 ml of benzoyl chloride, stirred under nitrogen at room temperature for about two hours, cooled in an ice bath again, treated with 1.65 ml of water and 1.7 ml of concentrated ammonium hydroxide, stirred under nitrogen at ambient temperature for about a half hour and then taken to dryness. The crude product was triturated with water followed by cyclohexane to give 0.4 g of 6-dibenzoyl-7-deaza-2'-deoxy-7-methyladenosine, 6.5 g (11.35 mmole) of which was hydrolyzed to the mono 6-benzoyl-7-deaza-2'-deoxy-7-methyladenosine by treatment with 200 ml of a 50% solution of 1N sodium hydroxide in ethanol and then acidifying with 2N hydrochloric acid. There was thus obtained 3.61 g (86%) of product, m.p. 172-175°C.

The latter (1.75 g, 4.75 mmole), in about 50 ml of dry pyridine, was treated with 1.86 g (5.23 mmole) of 4,4'-dimethoxytrityl chloride and the mixture stirred at ambient temperature under nitrogen for about four hours and 17 mL of methanol added then taken to dryness in vacuo. The product was purified by chromatography on silica gel, eluting the product with 3% methanol in chloroform. There was thus obtained 1.97 g (62%) of 6-benzoyl-7-deaza-2'-deoxy-7-methyl-5'-dimethoxytrityladenosine, m.p. 112-115°C.

A solution of 0.9 g (1.3 mmole) of the product in 7.5 ml of dry THF was treated with 1.0 ml (5.7 mmole) diisopropylamine and the solution treated dropwise with 1.0 ml (4.5 mmole) of chloro-β-cyanoethoxy-N,N-diisopropyl-aminophosphine over a period of about 40 minutes while stirring under nitrogen. The mixture was then stirred at ambient temperature under nitrogen for about 40 minutes and taken to dryness in vacuo to give the crude product which was purified by chromatography on silica gel, the product being eluted with helium saturated ethyl acetate. There was thus obtained 0.62 g (55%) of 6-benzoyl-7-deaza-2'-deoxy-7-methyl-3'-O-[(N,N-diisopropylamino)-β-cyanoethoxyphosphanyl]-5'-dimethoxytrityladenosine, m.p. 73-76°C.

examples, the letters A, G, W, X, C and T have the following nucleic acid base meanings:

A:	adenine
G:	guanine
W:	7-deazaadenine
X:	7-methyl-7-deazaadenine
C:	cytosine
T:	thymine

Table 1

Example	Structures (5'→3')
Control	AAA AAA AAA AAA AAA
Control	TTT TTT TTT TTT TTT
C-MYC-Sense	ATG CCC CTC AAC GTT
Antisense	AAC GTT GAG GGG CAT
1	AAA AAA AAA AAA AWA
2	AAA AAA AAA AAA WWA
3	AAA AAA AAA AAA AXA
4	AAA AAA AAA AAA XXA
5	AAA AAA AAA AAA XAA
6	XXC GTT GXG GGG CXT
7	CCT TCT CXG TCG GXT
8	WWC GTT GWG GGG CWT

The melting temperatures obtained for each of the oligomers described above are given in Table 2 below.

(a) The corresponding unmodified oligomer, CCT TCT CAG TCG GAC had a half life of 16 minutes.

730

The oligomer of Example 6 was found to inhibit translation to 13 ± 4% of control in the absence of RNase H and 5 ± 1% in the presence of RNase H in comparison with corresponding unmodified oligomer which inhibited
735 translation to 21 ± 4% of control in the absence of RNase H and 14% in the presence of RNase H.

3. An oligonucleotide according to Claim 2 wherein R'_2 is hydrogen.

4. An oligonucleotide according to Claim 2 wherein R'_2 is hydroxy.

5. An oligonucleotide according to Claim 3 containing from 12 to 24 bases.

6. An oligonucleotide according to Claim 5 containing 15 bases.

7. An oligonucleotide according to Claim 4 containing from 12 to 24 bases.

8. An oligonucleotide according to Claim 7 containing 15 bases.

9. An oligonucleotide according to Claim 6 wherein the modified purine nucleosides are incorporated within the three nucleotide units at either or both the 3'- and the 5'-ends of the oligomer.

10. An oligonucleotide according to Claim 8 wherein the modified purine nucleosides are incorporated within the three nucleotide units at either or both the 3'- and the 5'-ends of the oligomer.

11. An oligonucleotide according to Claim 6 wherein the modified purine nucleosides are incorporated within the three nucleotide units at either or both the 3'- and the 5'-ends or internally in the nucleotide sequence of the oligomer.

12. An oligonucleotide according to Claim 8 wherein the modified purine nucleosides are incorporated within the three nucleotide units at either or both the 3'- and the 5'-ends or internally in the nucleotide sequence of the oligomer.

INTERNATIONAL SEARCH REPORT

Inte. ...nal Application No
PCT/US 94/02996

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 286 028 (BOEHRINGER MANNHEIM GMBH) 12 October 1988 cited in the application see abstract see page 6, line 34 - page 7, line 13 ---	1-3,5,6, 15-17
X	NUCLEIC ACIDS RESEARCH. vol. 12, no. 23 , 11 December 1984 , ARLINGTON, VIRGINIA US pages 8939 - 8949 A.ONO ET AL. 'Synthesis of Deoxyoligonucleotides Containing 7-deazaadenine: Recognition and Cleavage by Restriction Endonuclease Bgl II and Sau 3AI (Nucleosides and Nucleotides Part 55).' see the whole document --- -/--	1,2,4, 15-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 June 1994	03. 08. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Scott, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMISTRY. vol. 29, no. 42 , 23 October 1990 , EASTON, PA US pages 9891 - 9901 P.C.NEWMAN ET AL. 'Incorporation of a Complete Set of Deoxyadenosine and Thymidine Analogues Suitable for the STudy of Protein Nucleic Acid Interactions into Oligodeoxynucleotides. Application to the EcoRV Restriction Endonuclease and Modification Methylase.' see the whole document ---	1-17
Y	BIOCHEMISTRY. vol. 29, no. 42 , 23 October 1990 , EASTON, PA US pages 9902 - 9910 P.C.NEWMAN ET AL. 'Interaction of the EcoRV Restriction Endonuclease with the Deoxyadenosine and Thymidine Bases in its Recognition Hexamer d(GATATC).' see the whole document ---	1-17
Y	BIOCHEMISTRY. vol. 26, no. 8 , 21 April 1987 , EASTON, PA US pages 2232 - 2238 F.SEELA ET AL. 'Palindromic Octa- and Dodecanucleotides Containing 2'-Deoxytubercidin: Synthesis, Hairpipn Formation, and Recognition by the Endodeoxyribonuclease EcoRI.' cited in the application see the whole document ---	1-17
Y	NUCLEIC ACIDS RESEARCH. vol. 16, no. 24 , 23 December 1988 , ARLINGTON, VIRGINIA US pages 11781 - 11793 A.FLIESS ET AL. 'Analysis of the Recofnition Mechanism Involved in the EcoRV Catalyzed Cleavage of DNA using Modified Oligodeoxynucleotides.' see the whole document --- -/--	1-17

INTERNATIONAL SEARCH REPORT

..nformation on patent family members

Inter nal Application No
PCT/US 94/02996

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU-B-	597483	31-05-90
		AU-A-	1439888	13-10-88
		CN-A-	88102038	26-10-88
		JP-A-	63275598	14-11-88
		ZA-A-	8802446	29-09-88